DECIPHERING THE URACIL GROWTH SENSITIVITY OF *Ashbya gossypii* PYRIMIDINE AUXOTROPHS

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INTRODUCTION

- The *A. gossypii Agura3* uracil auxotroph, which presents a riboflavin overproducing phenotype, is unable to grow at the same level of the parent strain when the medium is supplemented with uracil alone, being necessary the addition of uridine to overcome this effect[1].
- A similar effect was also reported for other flavinogenic fungi, such as *Pichia guilliermondii* (*Candida guilliermondii*)[2] and also for some species of the protozoan parasites *Leishmania*[3].
- The fluorinated analog of cytosine (5-Flucytosine; 5-FC) is a drug with antifungal properties commonly used against *Candida albicans*. Increased resistance to this drug by some strains was associated with a point mutation in the *FUR1* gene of the salvage pathway of pyrimidine ribonucleotides[4].
- Given the importance of uracil auxotrophic strains, it is of extreme pertinence to elucidate the determinants behind this inadequacy of uracil to rescue the growth of flavinogenic uracil auxotrophs.
- **In this work we investigated the suitability of intermediates of the pyrimidine ribonucleotides salvage pathway to rescue the growth of *A. gossypii Agura3*. Based on these results, we identified the reaction catalyzed by the enzyme encoded by the gene *AgFur1* (Fig. 1) as the potential limiting step of this pathway in *A. gossypii* and this hypothesis was tested by analyzing its resistance to 5-FC.**

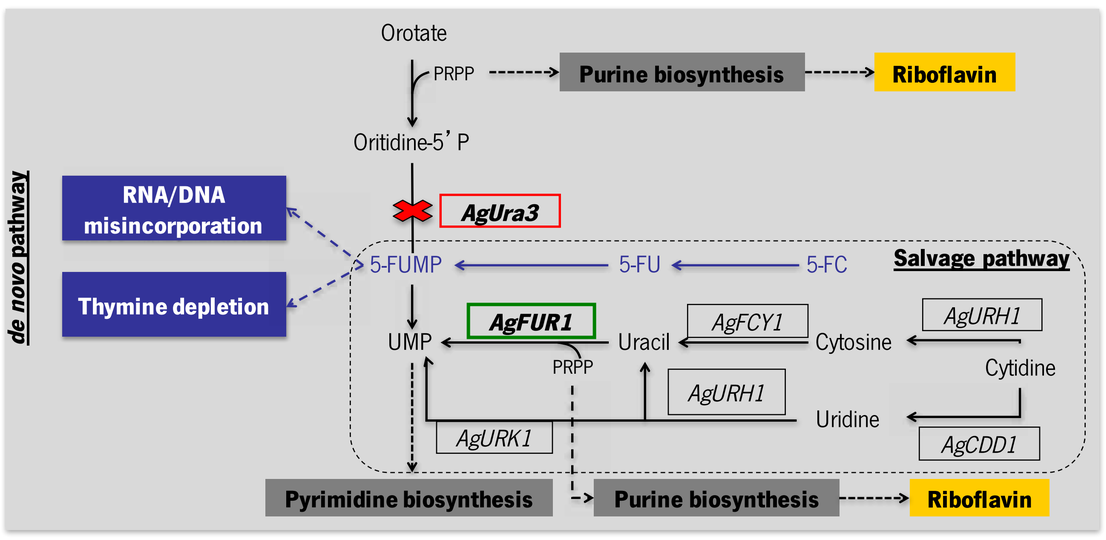
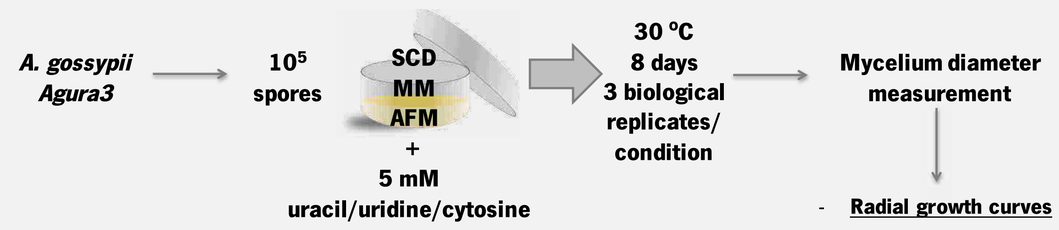


Figure 1 – Schematic representation indicating the *de novo* and salvage pathways of pyrimidine ribonucleotides. The × marks where the pyrimidine biosynthetic pathway is blocked in the *A. gossypii Agura3* uridine/uracil auxotrophic strain. In blue, the accepted mechanisms of action of the drug 5-Flucytosine (5-FC) and the respective deleterious effects for the cell. 5-FU, 5-Fluorouracil. Dashed arrows represent several reactions.

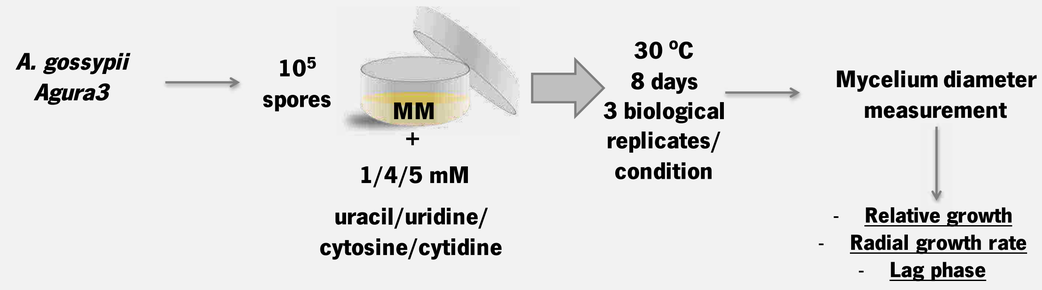
METHODS

1. RADIAL GROWTH CURVE OF *A. gossypii Agura3* STRAINS ON DIFFERENT MEDIA SUPPLEMENTED WITH DIFFERENT PYRIMIDINES AND PYRIMIDINE NUCLEOSIDES



- *A. gossypii Agura3* is an uracil/uridine auxotrophic strain derived from the reference strain *A. gossypii* ATCC10895.
- SCD, synthetic complete medium with amino acids and without uracil, uridine or cytosine (glucose, yeast nitrogen base (YNB) and $CaCO_3$); MM, minimal medium (glucose, YNB and $CaCO_3$); AFM, Ashbya Full Medium (glucose, tryptone, yeast extract and myo-inositol). All media contained agar.

2. GROWTH PARAMETERS OF *A. gossypii Agura3* GROWING ON MEDIA SUPPLEMENTED WITH DIFFERENT CONCENTRATIONS OF PYRIMIDINES AND PYRIMIDINE NUCLEOSIDES



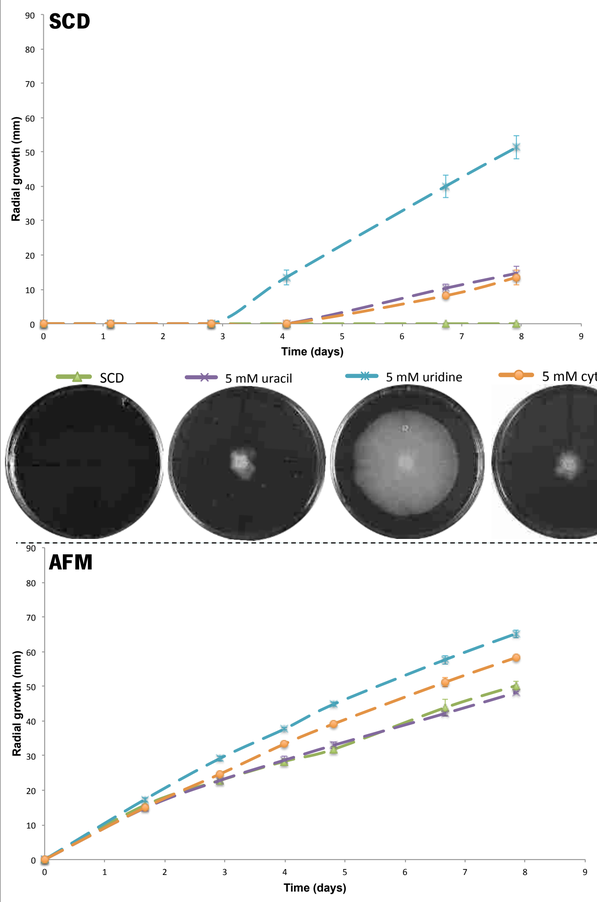
- The percentage of relative growth of *Agura3* strain was determined by normalizing the maximum radial growth of each condition to the growth in the condition that results in the highest growth (5 mM uridine).

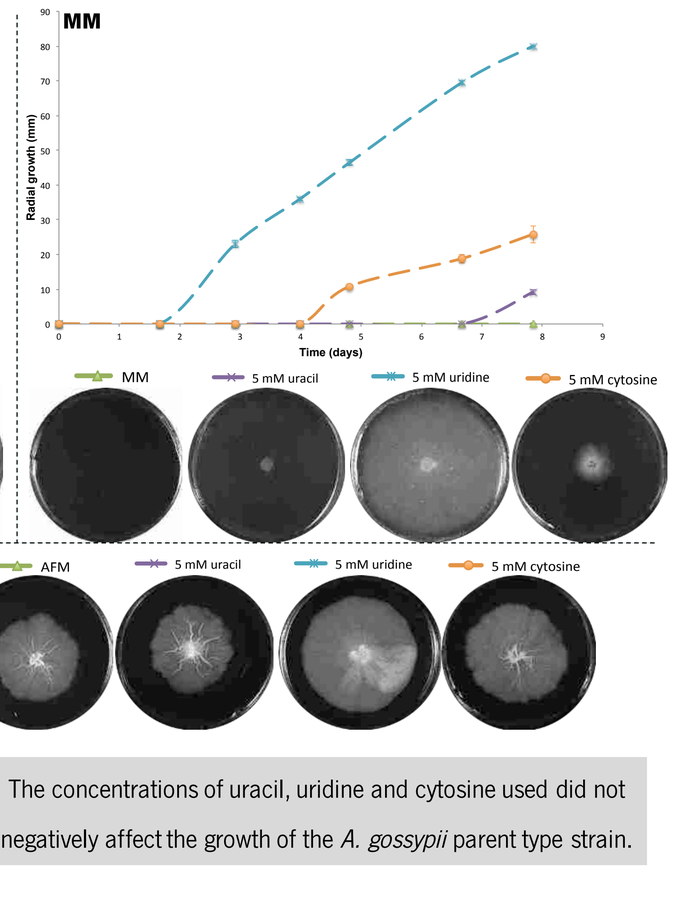
3. 5-FLUCYTOSINE RESISTANCE OF *A. gossypii* AND *S. cerevisiae* STRAINS TESTED BY SPOT DILUTION ASSAY

- Strains used: *A. gossypii* ATCC10895 and *S. cerevisiae* CENPK113-7D (wild type); *A. gossypii Agura3* and *S. cerevisiae* CENPK113-5D (uracil/uridine auxotrophic strains).
- Cells or spores were counted using a Neubauer Chamber and the 10^0 dilution concentration were defined to 10^6 spores or cells per mL. 10x serial dilutions were made until the 10^{-4} dilution. Subsequently, 10 µl from each dilution were inoculated on the plates.

RESULTS

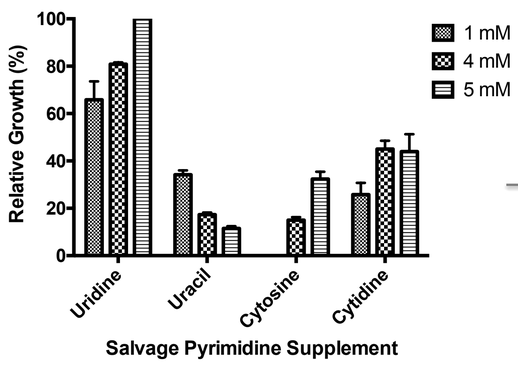
1. URIDINE CAN PROPERLY RESCUE THE GROWTH OF *A. gossypii Agura3* WHEREAS URACIL AND CYTOSINE CANNOT





The concentrations of uracil, uridine and cytosine used did not negatively affect the growth of the *A. gossypii* parent type strain.

2. THE BEST GROWTH PARAMETERS ACHIEVED BY *A. gossypii Agura3* ARE WITH SUPPLEMENTATION OF 5 MM URIDINE



Condition	Radial Growth Rate (mm/day; mean ± SD)	Lag Phase (days)
Uridine		
1 mM	10.5 ± 1.2	3
4 mM	10.8 ± 0.1	2
5 mM	13.3 ± 0.0	2
Uracil		
1 mM	5.5 ± 0.5	3
4 mM	2.8 ± 0.2	3
5 mM	–	7
Cytosine		
1 mM	–	8
4 mM	4.0 ± 0.3	5
5 mM	6.5 ± 0.6	4
Cytidine		
1 mM	6.9 ± 1.3	5
4 mM	7.2 ± 0.6	3
5 mM	7.0 ± 1.2	3

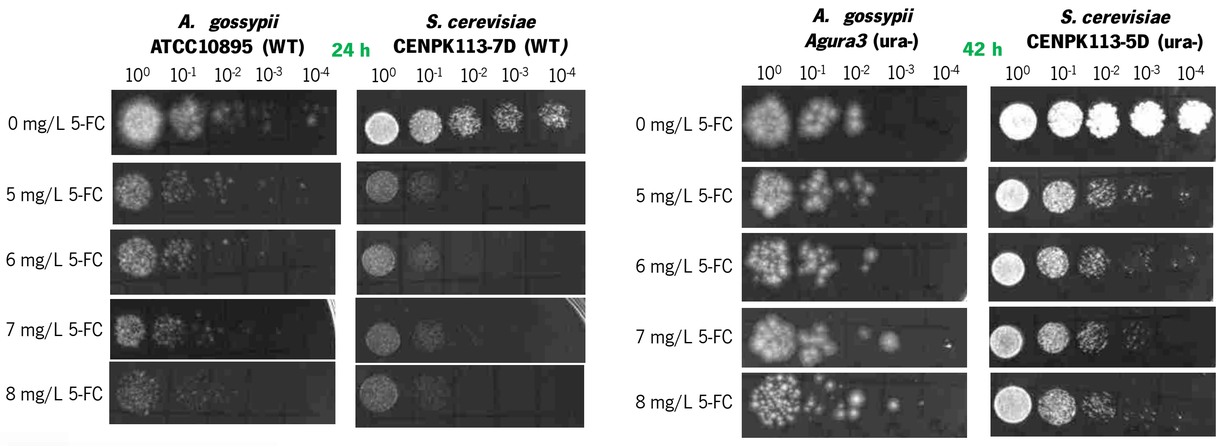
3. CONNECTING POOR UTILIZATION OF URACIL BY *A. gossypii Agura3* WITH 5-FC RESISTANCE VIA THE *AgFUR1* GENE

- Uridine can be metabolized directly to UMP via AgURK1p (Fig. 1) instead of passing the uridine → uracil → UMP reactions.
- On the other hand, uracil and cytosine must be metabolized through uracil → UMP reaction, while cytidine can take both routes.
- Therefore, the reaction catalyzed by AgFUR1p may possibly be the limiting step in *A. gossypii*. Especially because it requires PRPP, a precursor also necessary for the *A. gossypii*'s highly active purine biosynthetic pathway.
- Low activity of the enzyme involved in this reaction (AgFUR1p) can block the salvage pathway of pyrimidine ribonucleotides, leading to an accumulation of uracil and consequently impairment of the growth of *A. gossypii Agura3*.
- Since 5-FC needs to be metabolized in order to produce its effect, its toxicity is directly related with the activity of AgFUR1p.

Hypothesis

If the reaction catalyzed by the AgFUR1p is a limiting step,

***A. gossypii* should present increased resistance to the drug 5-FC**



CONCLUSIONS

- Uridine can properly rescue the growth of *A. gossypii Agura3* strain whereas uracil, cytosine and cytidine cannot. Therefore, uridine is the most adequate growth supplementation for *A. gossypii* uracil/uridine auxotrophic strains.
- Based on the results of the spot dilution assay, the growth of *A. gossypii* is apparently less affected by the drug 5-FC than that of *S. cerevisiae*, corroborating the hypothesis that the reaction catalyzed by the AgFUR1p is a limiting step in the salvage pathway of pyrimidine ribonucleotides in *A. gossypii*.
- Currently, we are performing the recombinant production, purification and molecular characterization of AgFur1p in order to further elucidate this phenotype of *A. gossypii* uracil/uridine auxotrophs and confirm our hypothesis.

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